

The in vivo Interaction of Streptococcal mAb10F5 in Lewis Rat Brains

An Honors Thesis (Honors 499)

by

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A handwritten signature in cursive script that reads "Dr. Marie Kelly-Worden". The signature is written in dark ink and is positioned below the printed name.

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Abstract

Group A streptococcal infection is being implicated in the formation of movement disorders such as PANDAS, Tourette syndrome, Sydenham's chorea, and general tics. It is suggested that antibodies produced against the conservative region of streptococcal M proteins are cross-reacting with neuronal tissue in an autoimmune response. One area of proposed cross-reactivity within the brain is the basal ganglia, the center for movement regulation. Antibodies against this region are called anti-basal ganglia antibodies. Monoclonal mouse antibody 10F5 (mAb10F5) is a streptococcal M6 antibody. Previous studies in our laboratory, using in vitro techniques, demonstrated that mAb10F5 bound in the basal ganglia of Lewis rats and has antiphospholipid properties. The current study sought to examine the interaction of mAb10F5 in Lewis rat brains in vivo. Rats were injected with either mAb10F5 or a positive control, myosin (type II) antibody, and euthanized after 24, 48, or 72 hours. Slices from the rostral and midrostral sections of these brains along with those of uninjected controls were analyzed using immunofluorescence and fluorescent microscopy. The caudate and putamen (CPu), a part of the basal ganglia, was significantly positive compared to controls at 24, 48, and 72 hours in the mAb10F5 treated group and at 24 and 48 hours in the myosin (type II) antibody treated group. It was discerned that in the mAb10F5 group the antibody crossed the blood-brain-barrier at 24 hours and remained in the CPu through 72 hours. The myosin (type II) antibody did not cross the blood-brain-barrier until 48 hours, and was no longer significantly in the CPu after 72 hours. These findings suggest that mAb10F5 is an anti-basal ganglia antibody and may be involved in movement disorders.

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Background

Group A streptococcal infections are commonly known to cause pharyngitis, impetigo, streptococcal toxic shock syndrome, necrotizing fasciitis, and septicemia (Cunningham 2000). Other sequelae now also being associated with group A streptococcal infection are acute rheumatic fever and a number of movement disorders such as Tourette syndrome, tics, pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS), and Sydenham's chorea (Husby et. al. 1976, Rizzo et. al. 2006, Swedo et. al. 1998, Kirvan et. al. 2003).

The emergence of neurological disorders such as PANDAS, Tourette syndrome, and Sydenham's chorea has been observed in patients weeks to months after streptococcal infection (Dale 2003, Swedo et al 1998). Tourette syndrome is described as having chronic multiple motor tics and one or more vocal tics, which may be episodic (American Psychiatric Association 2000, Rizzo et al. 2006). In comparison, PANDAS is characterized by the sudden appearance or exacerbation of tics or obsessive-compulsive disorder in 3-11 year olds following a group A streptococcal infection (Pavone et al. 2006, Swedo et al. 1998). These neuropsychiatric disorders, along with general tics, are in some cases thought to be caused by an autoimmune response to group A streptococcal infection (Dale 2003).

The cell membrane of group A streptococcus has M proteins attached to it which extend out ~50nm as alpha-helical coiled-coil dimers and are the bacteria's major virulence factor (Cunningham 2000, Jones et al.1988, Phillipps et al. 1981). The M protein contains amino acid repeat regions A, B, and C. The A repeat region is near the N terminus while the C region is closer to the carboxy-terminus (Bessin 1989). Regions near the N terminus are more varied, while sequences in the C region are conserved between M protein subtypes (Jones et al. 1988). More than 80 subtypes of streptococcal M protein have been identified (Cunningham 2000). These M protein subtypes have been divided into classes depending on their reactivity with

antibodies against the C repeat region. Class I M proteins contain epitopes within the C repeat region which react with M protein antibodies (Cunningham 2000, Bessin 1989).

The autoimmune activity thought to cause neurological disorders is explained by antibodies against several M proteins including the M6 protein, a class I M protein, of group A streptococcus displaying molecular mimicry. Certain M6 antibodies recognize the GLRRD sequence in the conserved C region of the M protein and cross-react with body tissues (Jones et al. 1986). Group A streptococcal antigens have been found to cross-react with a variety of mammalian tissues including cardiac and skeletal muscles, valvular tissue, kidney, skin, and neuronal tissues (Zabriskie 1967). In particular, M6 proteins have been shown to induce cross-reactive antibodies against neuronal tissues in rats (Dale 2003).

The presence of anti-brain antibodies is the most observed immunologic abnormality in patients with autoimmune neuropsychiatric disorders associated with group A streptococcal infection (Martino et al. 2004). Specifically, M6 antibodies have been found to cross-react with proteins in the basal ganglia more intensely than in cerebellar or cortical tissue (Bronze et al. 1993).

The basal ganglia is composed of the caudate and putamen (together the striatum), globus pallidus, subthalamus, and substantia nigra. After receiving information from the cerebral cortex, the basal ganglia facilitates wanted movement while inhibiting unwanted movement (Mink 2003). In the movement pathway, the caudate and putamen are normally silent while the globus pallidus sends signals to the thalamus to prevent movement. When a signal arrives from the cortex to the caudate and putamen it overrides the inhibition of the globus pallidus by ending the inhibitory signal. Antibodies against the basal ganglia are called anti-basal ganglia antibodies. Anti-basal ganglia antibodies have been found in patients with Tourette syndrome, obsessive-compulsive disorder, Sydenham's chorea, and PANDAS (Church et al. 2004, Rizzo et al. 2006, Singer et al. 2004, Trifiletti et al. 1999). In a study by Rizzo et al., antistreptococcal antibodies were found in 18 of 22 (82%) of Tourette syndrome patients with

anti-basal ganglia antibodies (Rizzo et al. 2006). Brain imaging, including volumetric studies, during the acute phase of PANDAS have distinctly shown an enlargement of the caudate and putamen (Dale 2003, Giedd et al. 2000, Peterson et al. 2000).

Monoclonal mouse antibody 10F5 (mAb10F5) is a group A streptococcal M serotype against M6 proteins (Jones et al. 1986). This serotype reacts with an epitope in the alpha-helical conserved region of the M6 protein (Jones et al. 1986, Jones et al. 1988). It is suspected of interacting with myosin in cardiac and brain tissue. Previous in vitro research in our lab has demonstrated that mAb10F5 has antiphospholipid characteristics. This research also found that in vitro mAb10F5 bound in regions of the basal ganglia, especially the caudate and putamen, in Lewis rats. The current study expanded upon our previous research and observes in vivo brain binding of mAb10F5 in Lewis rats.

Methods

The Lewis rat is the current animal model for streptococcal research (Li et al. 2004). Because of this, Lewis rats from our physiology colony were used. The rats were 9-12 months in age due to availability. Female Lewis rats were used since their lower weights would require the use of less antibody. The rats were kept in rectangular cages with wire lids, in groups of 2-3. They were kept on a twelve-hour light/dark cycle, and given constant access to chow (18% protein rodent diet) and water.

A total of 15 rats were used. The experimental group, injected with mAb10F5, and the experimental control group, injected with myosin type II antibody, each contained six rats. Three time points were examined (24, 48, and 72 hours), with two experimental animals and two experimental control animals per time point. The remaining three rats were used as uninjected negative controls.

A blood sample was taken from the rats prior to antibody injection (zero time point) and just before euthanization. This was done by first placing the rat in a restraining tube on a low

temperature heating pad. The rat was allowed to warm for approximately ten minutes to increase blood flow. The tip of the tail was cleaned and clipped with a razor blade. The tail was milked to obtain approximately 1ml of blood in a 1.3ml L-Heparin blood collection tube. The blood collection tube was kept on ice during this entire process. Once the blood was collected, bleeding was stopped by applying pressure. The blood sample was left on ice for 30-60 minutes as specified by the tube directions. It was then spun for 5 minutes at 1700rpm and 4°C in a refrigerated centrifuge. A pipette was used to remove the separated plasma and put it into a microcentrifuge tube. Blood and plasma samples were stored in a -80°C freezer for future immunoassays if necessary.

Rats were then weighed. Their individual average weights were recorded and used to determine their blood volume with an online blood volume calculator. Both mAb10F5 and the positive control myosin (Type II) antibody were administered at a 1:200 dose. The necessary amount of antibody was calculated using the blood volume. Antibody injections never exceeded 0.1ml.

The monoclonal mouse antibody 10F5 was supplied by Vincent Fischetti's laboratory. The myosin (type II) antibody was ordered from AbD Serotec. Each antibody was resuspended in sterile saline. This was done by first adding 400µl milli-Q water to the insert cup of an ultrafree-MC microcentrifuge filter and centrifuging it for 20 minutes at 2000xg and 22°C. The water was removed from both the insert cup and the bottom waste tube. The calculated amount of antibody was then added to the insert cup and centrifuged for 20 minutes at 2000xg and 22°C. The waste from the bottom tube was then removed, 50µl of sterile saline was added to the insert cup, and the filtering tube was centrifuged again for 20 minutes at 2000xg and 22°C. This step was repeated three times by adding 50µl again, then 30µl, and finally 30µl again of sterile saline. The remaining solution in the insert cup was brought back up to the original volume of antibody added using sterile saline.

For the injection of the antibody the rat was placed in a restraining tube. A tail vein was found and the area above it was cleaned. The calculated amount of antibody was injected into the tail vein using a 1ml syringe.

The rats were euthanized 24, 48, or 72 hours after injection. This was done by the physiology animal caretaker using carbon dioxide and a thoracotomy. The heads were then removed using a guillotine. The cerebrum was removed, rinsed with 1x phosphate buffer solution (PBS), and stored in 4% paraformaldehyde at -4°C .

For analysis, the brain was sliced using a Licor vibratome. To do this, the brain was first coronally quartered using a razor blade. Only the rostral and midrostral quarters were examined. The remaining caudal half of the brain was stored in 4% paraformaldehyde at -4°C for future observation. The quarter being sliced was attached to the slicing plate using superglue and then covered with PBS. Six to seven 90 μm slices were obtained and placed in the wells of a 24 well non-culture treated plate with 500 μl PBS. The remainder of the brain was once again stored in 4% paraformaldehyde at -4°C .

The brain slices were next prepared for immunofluorescence. First, the PBS was removed from the wells, 500 μl of PBS with 0.5% Triton X-100 was added, and the plate was set to rock for 30 minutes. Second, the solution was removed and the slices were washed for ten minutes with 500 μl 1x PBS. This wash was repeated two more times. The third wash was then removed, 200 μl of Odyssey blocking buffer was added to the wells, and the plate was set to rock for 30 minutes. A solution containing 250 μl 10x PBS, 2500 μl milli-Q water, 750 μl Odyssey blocking buffer, and 2.71 μl anti-mouse Alexa Fluor 488 was made. Of this, 300 μl was added to the wells and set to rock in the dark for 90 minutes. The solution was then removed and the slices were washed for ten minutes with 500 μl 1x PBS. This wash was repeated two more times. The PBS was removed from three of the wells, and the slices were covered with sudan black to decrease background fluorescence. This was set to rock for 30 minutes, then removed, and the slices were washed with 500 μl of 1x PBS. The entire preparation for

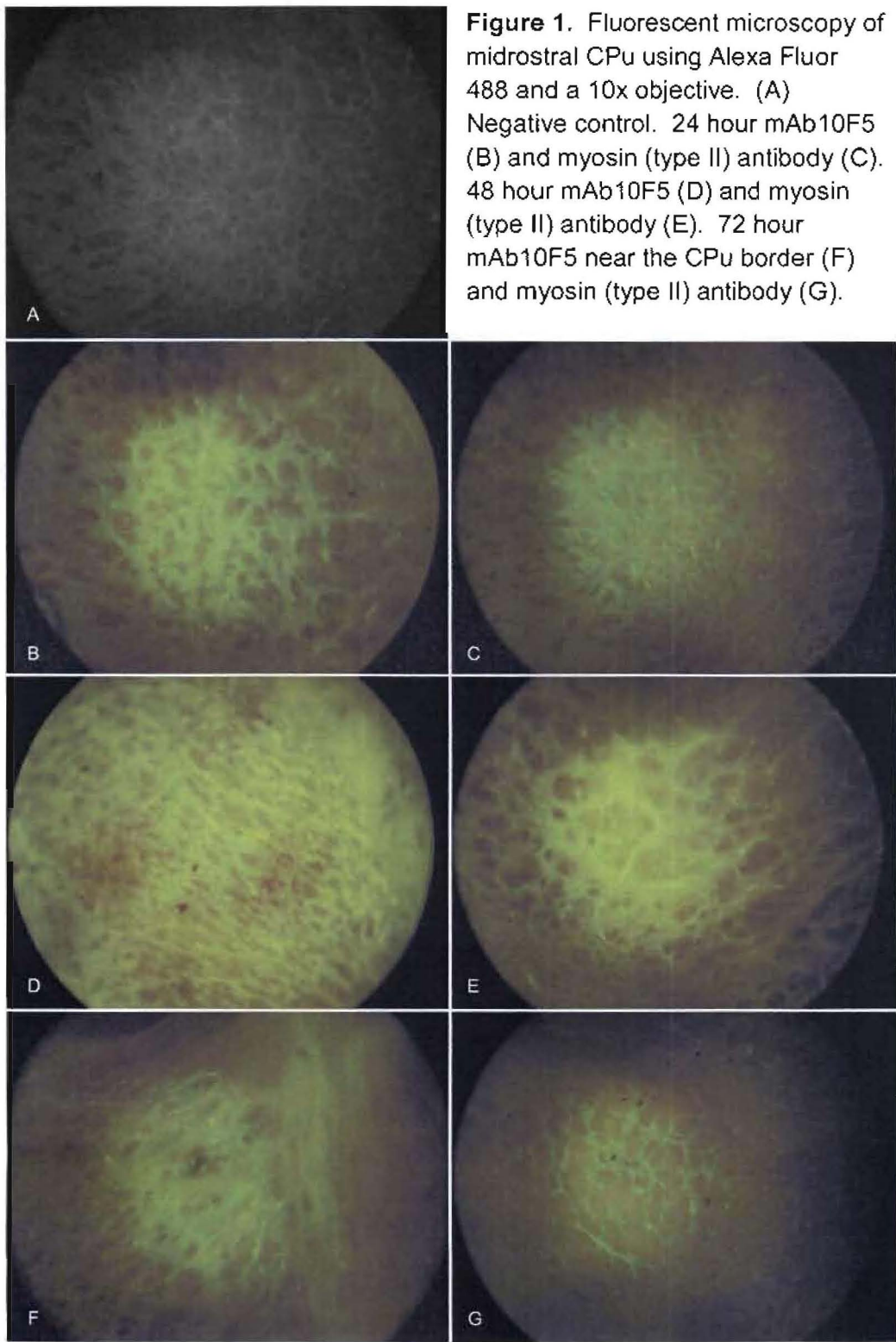
immunofluorescence was repeated using anti-mouse Alexa Fluor 350 on one slice from each quarter of the mAb10F5 brains, and anti-mouse Alexa Fluor 568 on one slice from each quarter of the positive control myosin (type II) and negative control brains. The second type of Alexa Fluor was used to eliminate false positives.

Brain slices were imaged using a Carl Zeiss fluorescent microscope with 5 and 10 x 0.25 NA CP-Achromat objectives. A Sony cybershot camera attached to the microscope was used to obtain fluorescent images of various regions including parts of the basal ganglia, the hippocampus, and the cortex. Image-Pro Express 6.0 was used to acquire fluorescent histograms with a range of 0-255. Fluorescence levels for each antibody were compared to the controls across the time points. A single factor ANOVA was used to find the significance ($p < .05$) of the data compared to control levels.

Results

In the rat brain, the caudate and putamen are combined to form the CPu. In the midrostral quarter, higher levels of fluorescence were observed in the CPu of both the mAb10F5 and the positive control myosin (type II) antibody treated rats compared to the uninjected negative controls (Figure 1). The uninjected controls had an average fluorescence of 62.81 on a scale ranging from 0-255 (Table 1). When compared to controls, at 24 hours mAb10F5 CPu had an average fluorescence of 124.02 and a p-value of 0.00437, while myosin (type II) antibody CPu had an average fluorescence of 95.09 and a p-value of 0.03953. At 48 hours, mAb10F5 CPu had an average fluorescence of 132.25 and a p-value of 0.00044, while myosin (type II) antibody CPu had an average fluorescence of 153.55 and a p-value of 0.00040. At 72 hours, mAb10F5 and myosin (type II) antibody CPu had fluorescent averages of 117.96 and 100.09 respectively, and p-values of 0.01132 and 0.05876. When compared to negative controls, CPu fluorescence levels for both the mAb10F5 and myosin (type II) antibody groups were significant at 24 and 48 hours, while only the values of the mAb10F5 group were

significant after 72 hours. One control slice did contain a false positive within the CPu. However, this outlier was not used in data analysis.



Fluorescence levels within the rostral CPu were also compared (Table 2). Average rostral CPu fluorescence for the uninjected controls was 91.09. Average CPu fluorescence at 24 hours was 91.61 for mAb10F5 treated rats and 77.67 for myosin (type II) antibody treated rats. At 48 hours, average CPu fluorescence was 85.78 for mAb10F5 treated rats and 111.29 for myosin (type II) antibody treated rats. Average fluorescence at 72 hours was 98.00 for mAb10F5 treated rats and 116.74 for myosin (type II) antibody rats. Since no pattern could be discerned from the data and images, further analysis was not performed.

Also in the midrostral CPu, a cloud of fluorescence was observed in the mAb10F5 treated rats usually at 24 hours, and in the myosin (type II) antibody treated rats usually at 48 hours (Figure 2). This phenomenon was not seen in the uninjected controls.

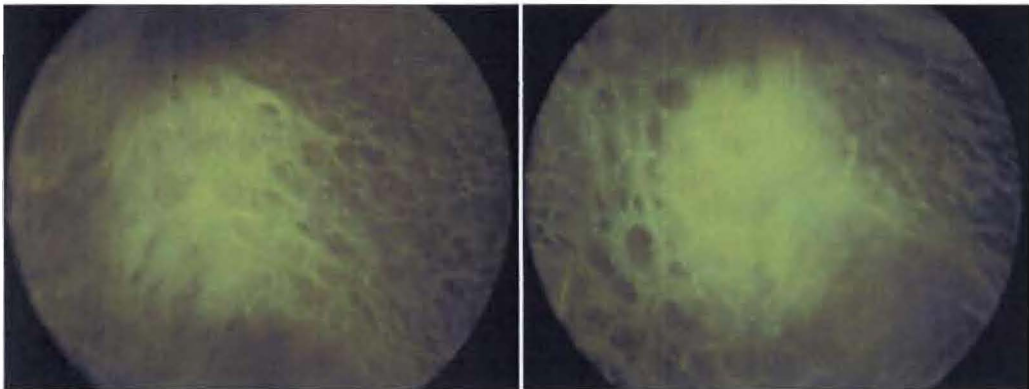
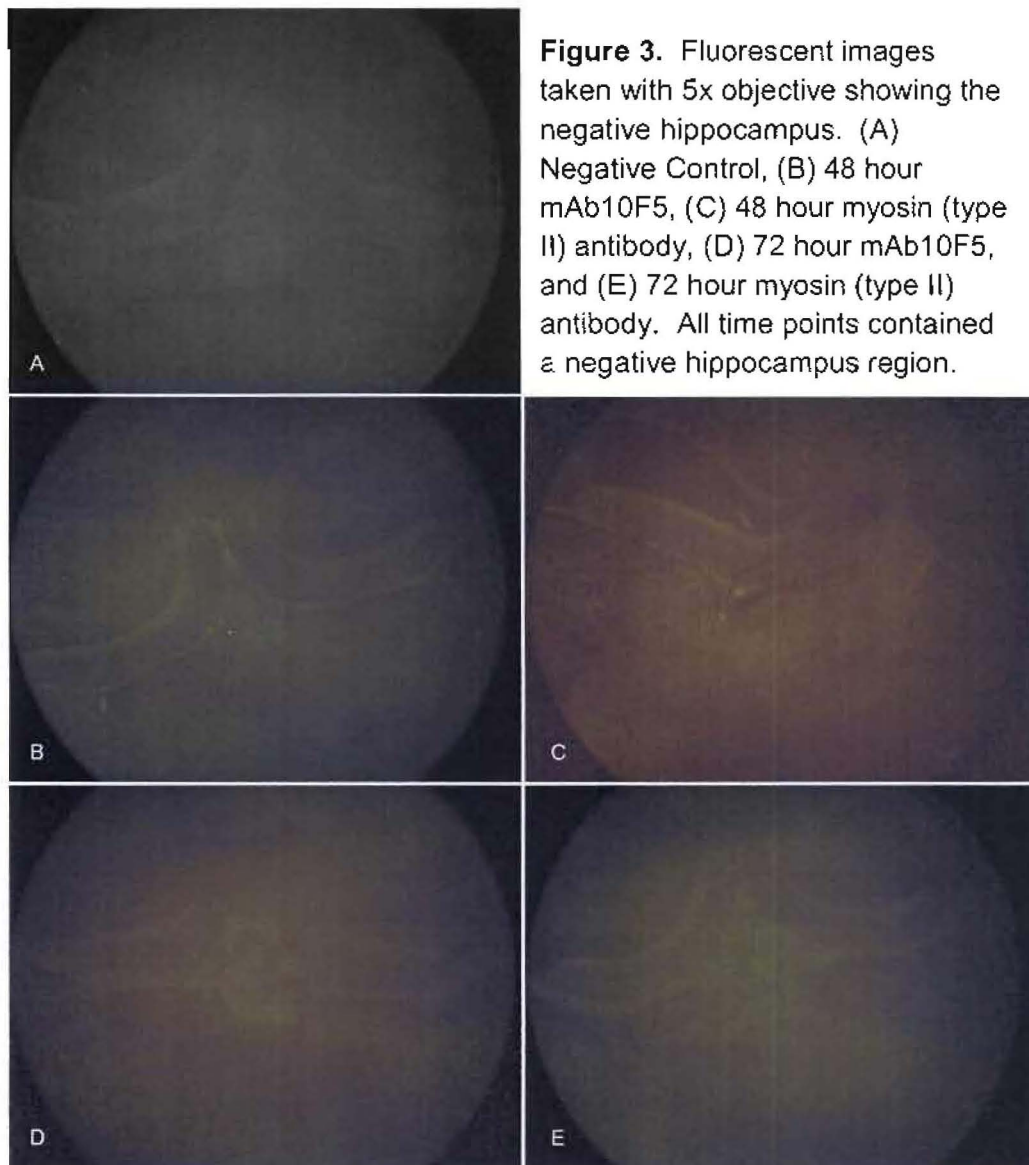


Figure 2. In the midrostral CPu, a cloud of fluorescence indicating the presence of antibody was observed in the mAb10F5 treated rats at 24 hours (left) and in the myosin (type II) antibody treated rats at 48 hours (right). This was not observed in the negative controls.

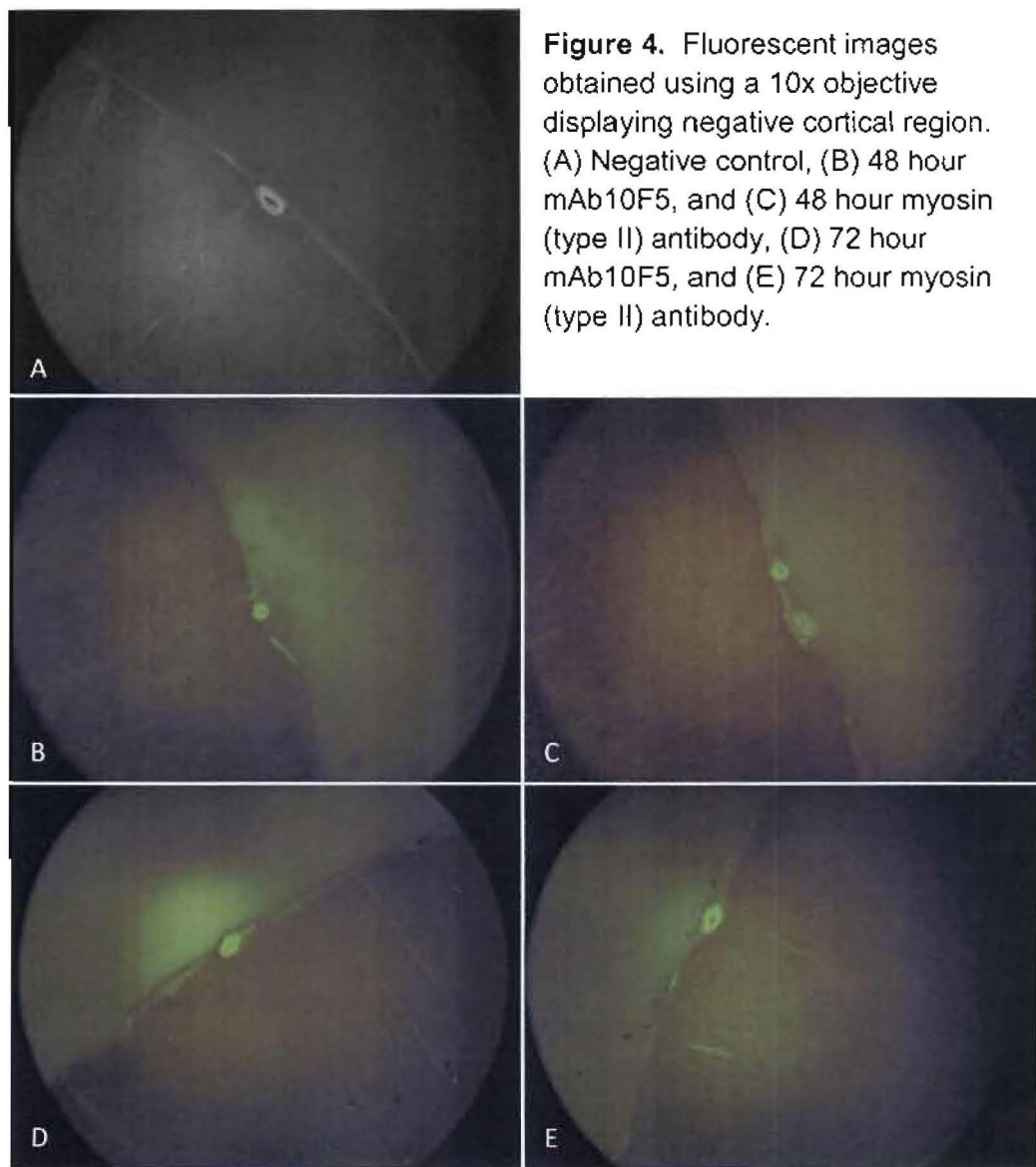
The hippocampus of both the mAb10F5 and myosin (type II) antibody treated groups was negative for all time points as compared with the uninjected controls (Figure 3). Data was analyzed from fluorescent images taken with a 5x objective as these represented each group at every time point (Table 3). However, data for the images taken with the 10x objective which were available were also negative. The hippocampus for the 48 and 72 hour mAb10F5, and the

24 hour myosin (type II) antibody treated groups were even significantly negative ($p<.05$) when compared to uninjected controls.



The cortex of rats injected with either mAb10F5 or myosin (type II) antibody was negative at all time points when compared to controls (Figure 4). Data from images in the same area of the midrostral cortex of antibody treated rats was compared to controls (Table 4). Values were taken from within the cortex and ignored the blood vessel (see figure 4). The

cortex of the negative control group had an average fluorescence of 72.70. The mAb10F5 treated group had an average fluorescence in the cortex of 76.39 at 24 hours, 86.16 at 48 hours, and 52.78 at 72 hours. The myosin (type II) antibody had cortex fluorescence values of 69.27 at 24 hours, 66.29 at 48 hours, and 69.52 at 72 hours. Neither the mAb10F5 group nor the myosin (type II) antibody group was significant for fluorescence in the cortex at any time point when compared to negative controls.



Discussion

Results from this study support the hypothesis from in vitro data that group A streptococcal antibody mAb10F5 binds in the area of the basal ganglia in the brains of Lewis rats. This finding is congruent with the in vitro study done previously in our lab. The mAb10F5 was found significantly in the CPu but not the hippocampus or cortex as determined by the presence of fluorescence. This suggests that mAb10F5 is an anti-basal ganglia antibody. As anti-basal ganglia antibodies have been documented in PANDAS, Tourette syndrome, and Sydenham's chorea, a 10F5e antibody could be the culprit in these movement disorders (Church et al. 2004, Rizzo et al. 2006, Singer et al. 2004, Trifiletti et al. 1999). This means that mAb10F5, which recognizes the conserved region of the streptococcal M6 protein, is cross-reacting with neuronal tissues in an autoimmune fashion.

Though we did not monitor the rats for changes in motor activity for this study, we can hypothesize how a change may occur. The mAb10F5 bound specifically in the CPu, illustrating that it most likely works by inhibiting the inhibition of the globus pallidus. Such dysfunction would allow the thalamus to send unwanted movement signals to the motor cortices to be executed. Since our laboratory has previously observed that mAb10F5 has antiphospholipid properties, the streptococcal antibody may be interacting with endothelial or pericyte cells at the level of the brain capillaries. Once inside the CPu, an interaction involving components of the CPu could promote neurotransmitter release and dysfunction of movement regulation leading to movement disorders.

In the midrostral CPu, mAb10F5 treated rats had fluorescence levels significantly ($p < .05$) elevated above negative control values at all three of the time points (24, 48, and 72 hours). The cloud of fluorescence, usually visualized at 24 hours, may be the antibody leaking out of the capillaries into the interstitial fluid and the entrapment of Alexa Fluor in the interstitial space. Since the fluorescence levels continue to be significantly elevated at 48 hours, the antibody has

most likely been taken up into the tissues by this time point. At 72 hours, the antibody remained in the tissues of the CPu demonstrated by the presence of fluorescence still significantly above negative control values.

In comparison, the midrostral CPu fluorescence levels of the myosin (type II) antibody treated rats were only significantly elevated above controls during the 24 and 48 hour time points. The cloud of antibody as it diffused into the interstitial fluid was usually not seen until the 48 hour time point. By 72 hours the fluorescence levels dropped to an insignificant value. This suggests that the myosin (type II) antibody was beginning to be removed from the tissues by 72 hours.

Looking at the activities of the antibodies, it can be seen that both mAb10F5 and myosin (type II) antibody can pass through the blood-brain-barrier. This tended to happen at 24 hours in the mAb10F5 rats, but not until 48 hours in the myosin (type II) antibody rats. Both antibodies had very high fluorescence values at 48 hours, showing that the antibodies were predominantly in the CPu. At 72 hours, the myosin (type II) antibody was already beginning to be removed from the area, while mAb10F5 remained in the CPu.

The data gathered in the CPu of the rostral quarter was inconclusive. Many of the blood vessels within the Lewis rat brain come up past the rostral section into the midrostral section making capillary beds denser in the midrostral region. Therefore, the lack of sufficient data in the rostral quarter may not be because mAb10F5 is incapable of binding in this area. It may simply be that the antibody has more chances to cross the blood-brain-barrier in the midrostral region, due to the slower movement of blood through the capillaries, than it does in the rostral area.

Conclusion

The group A streptococcal M6 antibody, mAb10F5, cross reacts with tissues in the basal ganglia making it an anti-basal ganglia antibody. The presence of the antibody in the CPu

suggests that mAb10F5 is crossing the blood-brain-barrier to bind to these neuronal tissues. Such binding could allow mAb10F5 to disrupt the normal function of the CPU and inhibit the inhibition of unwanted movement. This type of autoimmune interaction would implicate mAb10F5 like antibodies as a potential cause of movement disorders such as PANDAS, Tourette syndrome, and Sydenham's chorea. Future studies need to analyze patients with these disorders for 10F5e antibodies. Also, further insight into the exact mechanism of mAb10F5 action is necessary.

It was observed that the mAb10F5 diffused out of the capillaries and into the tissues of the CPU at 24 hours and remained there through 72 hours. In contrast, myosin (type II) antibody did not diffuse into the interstitial fluid until 48 hours, and was beginning to be removed from the CPU by 72 hours. Additional time points are necessary to establish the amount of time it takes for mAb10F5 to be removed. Longer animal studies, possibly with increased or multiple mAb10F5 doses, would be needed. Future studies would need to be performed to determine if neurological symptoms manifest from the presence of the antibody in the brain. A correlation of symptoms with indicators of PANDAS, Tourette syndrome, and/or Sydenham's chorea would elucidate which disorders mAb10F5 plays a role in.

Appendix

Group		Fluorescent Levels						Average p-value	
Neg. Control		49.472	78.504	84.685	60.981	21.534	81.664	62.807	
24 hour	mAb10F5	88.387	115.012	107.113	156.803	147.776		124.018	0.00437
	antimyosin	72.050	107.319	88.038	120.822	87.206		95.087	0.03953
48 hour	mAb10F5	110.187	134.460	122.819	139.918	153.889		132.255	0.00044
	antimyosin	165.475	161.870	180.212	160.014	100.190		153.552	0.00040
72 hour	mAb10F5	99.047	136.498	118.339				117.961	0.01132
	antimyosin	136.792	94.597	102.791	66.168			100.087	0.05876

Table 1. Midrostral CPu fluorescence levels on a scale from 0-255. Obtained from a single factor ANOVA, p<.05 values indicate significant binding of the antibody at the time point compared to the negative control group. Both the mAb10F5 group and the myosin (type II) antibody group had significant fluorescence at 24 and 48 hours, while only the mAb10F5 group's values were significant at 72 hours.

Group		Fluorescent Levels						Average	
Neg. Control		106.558	46.183	69.145	100.801	111.914	106.139	96.899	91.091
24 hour	mAb10F5	94.277	62.906	58.654	87.520	151.112	95.185		91.609
	antimyosin	36.426	93.204	50.548	133.955	74.195			77.666
48 hour	mAb10F5	86.809	86.480	84.050					85.780
	antimyosin	116.817	181.795	120.567	80.708	56.567			111.291
72 hour	mAb10F5	62.708	117.278	121.104	110.453	78.440			97.996
	antimyosin	139.606	104.455	128.943	78.519	132.176			116.740

Table 2. Rostral CPu fluorescence levels. Results showed no patterns, making further analysis unnecessary.

Group		Fluorescent Levels				Average p-value	
Neg. Control		54.377	51.580	50.667		52.208	
24 hour	mAb10F5	61.159	50.766			55.963	0.43055
	antimyosin	39.022				39.022	0.02748
48 hour	mAb10F5	46.709	43.302			45.005	0.03310
	antimyosin	50.661	51.146			50.904	0.43526
72 hour	mAb10F5	34.194				34.194	0.01501
	antimyosin	51.580	40.401	46.713	42.487	45.295	0.07418

Table 3. Fluorescence levels of the hippocampus. Obtained from a single factor ANOVA, $p>.05$ values show that fluorescence levels were not significant when compared to negative controls. Those values that are significant ($p<.05$) only demonstrate that the fluorescence levels were significantly negative compared to the negative controls. These results indicate that the hippocampus was negative for antibody binding in both the mAb10F5 and myosin (type II) antibody groups.

Group		Fluorescent Levels					Average p-value	
Neg. Control		73.380	121.425	54.791	58.769	55.155	72.704	
24 hour	mAb10F5	53.164	68.788	107.204			76.385	0.86363
	antimyosin	84.270	54.265				69.268	0.88508
48 hour	mAb10F5	122.109	50.201				86.155	0.65631
	antimyosin	60.072	66.730	45.356	93.002		66.290	0.71387
72 hour	mAb10F5	52.161	60.475	45.697			52.778	0.28929
	antimyosin	71.734	64.899	71.923			69.519	0.85706

Table 4. Levels of fluorescence in the midrostral cortex. All values were insignificant ($p>.05$) when compared to the negative control group. This indicates that the cortical region was negative at all time points for both mAb10F5 and myosin (type II) antibody.

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